**Open Literature Review Summary**

**Chemical Name:** Imidacloprid

**PC Code:** 129099

**ECOTOX Record Number and Citation:**

MRID Number: 47523410

Decourtye, A., E. Lacassie, and M.H. Pham-Delegue. 2003. Learning performances of honeybees (*Apis mellifera* L) are differentially affected by imidacloprid according to the season. Pest Manag Sci 59: 269-278.

**Purpose of Review (DP Barcode or Litigation):** N/A

**Date of Review:** 12/03/13

**Summary of Study Findings:**

*Summary:* Acute and chronic oral tests were conducted on caged honeybee workers using imidacloprid and its metabolite (5-OH-imidacloprid) under laboratory conditions. The metabolite 5-hydroxy imidacloprid showed a 48-hr oral LD50 value (153 ng/bee) five times higher than that of imidacloprid (30 ng/bee). Chronic feeding tests indicated that the lowest observed effect concentration of imidacloprid and 5-hydroxy on mortality of winter bees were 24 and 120 µg/kg, respectively. These two chemicals were also evaluated using the proboscis extension reflex at two periods of the year. The LOEC of imidcacloprid in summer bees was 12 µg/kg and 48 µg/kg for winter bees. Both parent imidacloprid and the 5-hydroxy metabolite reduced learning performance.

*Methods:* The LD50 of imidacloprid and 5-OH-imidacloprid were determined using method No. 95 for risk assessment of pesticides to bees from the 'Commission des Essais Biologiques'. The study authors modified the design by mixing worker bees from three different hives in order to reduce the potential for a hive effect.

Technical grade imidacloprid and 5-OH-imidacloprid (both 99.4% pure) were used. Imidacloprid of 98% purity was also obtained. Dimethoate of 96% purity was obtained as well. Five concentrations of imidacloprid and 5-OH-imidacloprid were used ranging from 0.2 to 3.2 mg/L for imidacloprid, and from 1.25 to 20 mg/L for 5-OH-imidacloprid, in a geometrical progression of factor 2. Dimethoate was used as a positive control at doses of 0.10 and 0.35 µg/bee.

Stock solutions of each chemical were prepared in acetone. The chemicals were added to a sucrose solution (500g/L). The final concentration of acetone in the sucrose solutions was 10 ml/L. The effects of insecticide-spiked solutions were compared with that of an untreated sucrose solution containing 10ml/L acetone. No negative control was used in the study.

Tests were carried out with *Apis mellifera* worker bees of unknown age. Bees from frames without a brood, to avoid the youngest bees, were caged in groups of 20. They were provided with a sugar solution and incubated (darkness, 25 (±2)ºC, 40 (±10)% RH)

overnight. The three colonies appeared to be in good health based on hive health tests for diseases.

The toxicity tests were conducted on late summer bees (August to October). Three replicates for each of the five concentrations and of the untreated control were undertaken simultaneously, and this was repeated at least three times over the experimental period. Twenty bees were used per replicate. After a 2hr starvation period, each group of 20 bees received 200 µL of the treated or the control sugar solution, in daylight and at 25 (±2)ºC. After the consumption of the 200µL of sugar solution (10µL per bee), the bees were put back into an incubator (darkness, 25 (±2)ºC, 40 (±10)% RH) and provided with an untreated sugar solution *ad libitum*. Mortality was recorded 48hrs after the beginning of the treatment. In order to calculate the LD50 values, mortality rates of treated groups were corrected using Abbott's formula. LD50 values were calculated with a probit regression analysis.

Frozen samples of contaminated sucrose solution at the concentrations delivered to the bees were analyzed for residues. Control untreated samples of sucrose solution were used as a blank matrix to prepare matrix matched standards for calibration. Liquid chromatography-mass spectrometry/mass spectrometry method was used. The LOD and LOQ for imidacloprid ranged from 1 to 2µg/kg, and from 5 to 10µg/kg for 5-OH-imidacloprid.

For the proboscis extension reflex (PER) assay, in the first experiment imidacloprid and 5-OH-imidacloprid were tested at six concentrations (from Bayer labs at 99.4% purity), with a geometrical progression of factor 2 (1.5 - 48µg/kg for imidacloprid and 7.5 – 240 µg/kg for 5-OH-imidacloprid). In the second experiment, a range of seven concentrations of imidacloprid was used (from Cluzeau labs at 98% purity): 1.5 – 96µg/kg. All solutions were made up as 500g/L sucrose, with 10 ml/L acetone. The control and contaminated sucrose solutions were kept at -20 (±1)ºC (from 1 – 15 days) and defrosted at ambient temperature, in natural daylight, before use.

**Commented [JPD1]:** Photodegradation?

PER experiments were carried out with worker bees of *Apis mellifera ligustica*. Experiment 1 used 'winter bees' (December to February). These bees were collected from hives maintained in a heated apiary (25 (±5)ºC). Even during the overwintering period, the queens maintained under heated conditions continued to lay eggs. Experiment 2 was conducted using summer bees in July, with bees collected from outdoor hives, when the foraging activity of the workers and queen activity were high. Honeybees of unknown age were used. Emerging workers were caged in groups of 60 individuals. They were provided with sugar food (mixture of sugar and honey) and water *ad libitum* during the initial 2 days, and with pollen for the following 8 days. After 2 days, the insecticide-treated sugar solutions were provided. The bees were kept in an incubator (33 (±2)ºC, 40(±10)% RH, darkness). The rearing temperature applied is higher than that recommended in the standard acute toxicity method (25 (±2)ºC), but according to the authors corresponds to the hive temperature. The exposure lasted until the bees were 14 – 15 days old, and the bees were then used in the PER assay.

In experiment 1, every testing day included bees previously exposed to three concentrations of imidacloprid and of its main metabolite were tested, as well as untreated control bees, leading to a total of 60 – 70 bees tested per day, with 5 – 6 bees

for each treatment. This was done repeatedly, until about 30 bees per treatment were obtained. It was not possible to test all six concentrations of the chemicals daily, so three concentrations were tested in a first set, then the three remaining ones in a second set of tests. In experiment 2, the bees subjected to prior exposure to the seven concentrations of imidacloprid, and the untreated control bees, were tested daily. This was repeated until the samples of tested bees reached approximately 30 individuals per treatment.

After treatment, the bees were mounted individually in glass tubes with only their antennae and mouthparts free. They were starved for 4hrs before conditioning. The bees were selected for showing a proboscis extension reflex after stimulation of the antennae with a sucrose solution. The bees were familiarized with the mechanical stimulation and with the experimental background. For the conditioning trials, the conditioned stimulus (10µL of pure linalool soaked on a filter paper strip inserted in a Pasteur pipette cartridge) was delivered for 6 s. During odor delivery, the PER was elicited after 3 s by contacting the antennae with a sucrose solution as the unconditioned stimulus, and the same solution was immediately given as a reward, before the odor delivery ended. Three conditioning trials were carried out with 20-30 min inter-trial duration. The individuals were then subjected to one test trial, the conditioned stimulus being delivered for 6 s.

For analysis, the mean amounts of sugar solution consumed daily over the 11 days of treatment prior to PER testing were compared among the concentrations (including the untreated group). The mortality cumulated over 11 days of treatment with each chemical was compared between every concentration and the control. For each chemical, the number of initial reflex responses and the number of conditioned responses in the test trial were compared between each concentration of the chemical and the control.

*Results:*

Acute toxicity: mortality in the dimethoate treatment ranged from 50% to 100% and the mortality in the untreated control was less than 10%. LD50 values determined 48hrs after the oral treatments are presented in **Table 1**. Imidacloprid showed an LD50 value of 30.6 ng/bee, and 5-OH-imidacloprid showed an LD50 of 153.5 ng/bee.

**Table 1.** Acute oral toxicity of imidacloprid and 5-OH-imidacloprid in honeybees

| *Chemical* | *48-h $LD_{50}$[a] (ng per bee)* | *95% CL* | *Slope* |
|---|---|---|---|
| Imidacloprid | 30.6 | 26.7–36.3 | 2.21 |
| 5-OH-imidacloprid | 153.5 | 125.9–196.9 | 0.88 |

[a] Tests were performed according to CEB testing guideline No 95.[27] $LD_{50}$ values were calculated using log-probit analysis.[30] The number of bees per group was between 180 and 360.

Nominal and dosed concentrations of imidacloprid and 5-OH-imidacloprid in the sucrose solutions are given in **Table 2**. For imidacloprid containing solutions, the rate of recovery between nominal and dosed concentrations ranged from 103% at 48µg/kg to 213% at 1.5µg/kg, possibly due to evaporation of the solvent during syrup preparation. Traces of the olefin metabolite were found in solutions containing imidacloprid at 6 and 24µg/kg. The rate of recovery for 5-OH-imidacloprid ranged from 70% at 240µg/kg to

113% at 30µg/kg. The olefin was also found in these latter test solutions. Percent mortality is shown for the various experiments on chronic toxicity of imidacloprid and 5-OH-imidacloprid in **Table 3** and **Table 4**. Note that the percent mortality in the control for 5-OH-imidacloprid is relatively high at 17.2%.

**Table 2.** Nominal and dosed concentrations ($\mu g kg^{-1}$) of (A) imidacloprid-containing solutions and (B) 5-OH-imidacloprid-containing solutions as used in Experiment 1

| | *Nominal* | *Dosed* | *Nominal* | *Dosed* |
|---|---|---|---|---|
| A | *Imidacloprid* | | *5-OH-imidacloprid* | |
| | 0 | <LOD[a] | 0 | <LOD |
| | 1.5 | 3.2 | 0 | <LOD |
| | 6 | 8.8 | 0 | <LOD |
| | 24 | 32.8 | 0 | <LOD |
| | 48 | 49.5 | 0 | <LOD |
| B | *5-OH-imidacloprid* | | *Imidacloprid* | |
| | 0 | <LOD | 0 | <LOD |
| | 7.5 | <LOQ[b] | 0 | <LOD |
| | 30 | 34.1 | 0 | <LOD |
| | 120 | 83.8 | 0 | <LOD |
| | 240 | 168.4 | 0 | <LOD |

[a] Limit of detection (LOD): $1 \mu g kg^{-1}$ for imidacloprid, $5 \mu g kg^{-1}$ for 5-OH-imidacloprid.
[b] Limit of quantification (LOQ): $2 \mu g kg^{-1}$ for imidacloprid, $10 \mu g kg^{-1}$ for 5-OH-imidacloprid.

**Table 3.** Chronic oral toxicity of imidacloprid in honeybees

| | *Experiment* | *Nominal concentrations ($\mu g kg^{-1}$)* | *Mortality (%)[c]* |
|---|---|---|---|
| A | Experiment 1 (winter bees)[a] | Control | 11.6 |
| | | 1.5 | 12.7 |
| | | 3 | 3.0 |
| | | 6 | 9.4 |
| | | 12 | 11.1 |
| | | 24 | 16.1 |
| | | 48 | 20.5* |
| B | Experiment 2 (summer bees)[b] | Control | 3.3 |
| | | 1.5 | 8.3 |
| | | 3 | 8.3 |
| | | 6 | 5 |
| | | 12 | 7.2 |
| | | 24 | 7.7 |
| | | 48 | 9.4 |
| | | 96 | 17.7† |

[a] The number of bees per group was between 180 and 360.
[b] The number of bees per group was 180.
[c] In each experiment, the cumulative mortalities in treated groups and in the control group were compared using chi-squared test with 1 *df*.
* $P < 0.0083$.
† $P < 0.0071$.

**Table 4.** Chronic oral toxicity of 5-OH-imidacloprid in winter honeybees[a]

| *Nominal concentrations ($\mu g kg^{-1}$)* | *Mortality (%)*[b] |
|---|---|
| Control | 17.2 |
| 7.5 | 3.3 |
| 15 | 13.3 |
| 30 | 19.4 |
| 60 | 10.5 |
| 120 | 26.6 |
| 240 | 41.0* |

[a] The number of bees per group was 180.
[b] In each experiment, the cumulated mortalities in treated groups and in the control group were compared using chi-squared test with 1 *df*.
* $P < 0.0083$.

PER assay: The syrup consumption rate per day of winter bees over the 11 day treatment period was calculated to be 28.8 to 33.7µl per bee per day, and the consumption was not statistically different from the control groups. The consumption of 5-OH-imidacloprid treated syrups was significantly lower than that of the control groups at 240, 120, and 30 µg/kg. Cumulative mortality in exposed bees at 1.5 – 24 µg/kg did not differ significantly from the control bees; however, a significant increase in mortality was observed at 48µg/kg (20.5% versus 11.6% mortality after 11 days, in the treated and control group respectively). The number of dead bees exposed to 240µg/kg of 5-OH-imidacloprid was significantly different from the control group.

Reflex response in imidacloprid-treated and untreated winter bees was shown to be the same (**Table 5**). Reflex response levels obtained after treatment with 5-OH-imidacloprid solutions at concentrations greater than 30µg/kg were significantly lower than the control (**Table 6**). **Figures 2**, **3, and 4** show the olfactory learning performances represented as the percentage of conditioned PER obtained at the test trial following the training procedure in bees fed the six concentrations of imidacloprid or 5-OH-imidacloprid and in the control bees. Only bees fed 48µg/kg were significantly different. The 120 and 240µg/kg concentrations of 5-OH-imidacloprid induced statistically different responses relative to the control group.

The consumption of sugar solutions contaminated with all seven concentrations of imidacloprid by summer bees were equivalent to the consumption of control sugar solution. The cumulative mortality recorded in summer bees was significantly increased in the presence of imidacloprid at 96µg/kg only. Concentrations of 48 and 96µg/kg of imidacloprid elicited a significant decrease in the level of reflex responses compared with the control. Treatment with imidacloprid to summer bees affected olfactory learning performances at 12, 24, 48, and 96 µg/kg.

**Table 5.** Effects of imidacloprid on reflex responses in honeybees

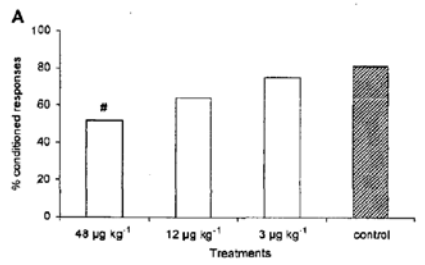
| Experiment | Nominal concentrations ($\mu g kg^{-1}$) | Reflex responses (%)[c] |
|---|---|---|
| A Experiment 1 (winter bees)[a] | Control | 52.4 |
| | 1.5 | 60.0 |
| | 3 | 44.7 |
| | 6 | 60.0 |
| | 12 | 55.0 |
| | 24 | 42.0 |
| | 48 | 36.6 |
| B Experiment 2 (summer bees)[b] | Control | 90.1 |
| | 1.5 | 81.9 |
| | 3 | 85.6 |
| | 6 | 78.6 |
| | 12 | 83.6 |
| | 24 | 80.0 |
| | 48 | 59.0* |
| | 96 | 69.7* |

[a] The number of bees per group was between 68 and 163.
[b] The number of bees per group was between 60 and 66.
[c] In each experiment, the number of reflex responses in treated groups and in the control group were compared using Chi-square test with 1 *df*.
* $P < 0.0071$.

**Table 6.** Effects of 5-OH-imidacloprid on reflex responses in winter honeybees[a]

| Nominal concentrations ($\mu g kg^{-1}$) | Reflex responses (%)[b] |
|---|---|
| Control | 61.5 |
| 7.5 | 55.3 |
| 15 | 57.5 |
| 30 | 52.8 |
| 60 | 40.0* |
| 120 | 29.3* |
| 240 | 21.4* |

[a] The number of bees per group was between 56 and 156.
[b] In each experiment, the number of reflex responses in treated groups and in the control group were compared using chi-squared test with 1 *df*.
* $P < 0.0071$.



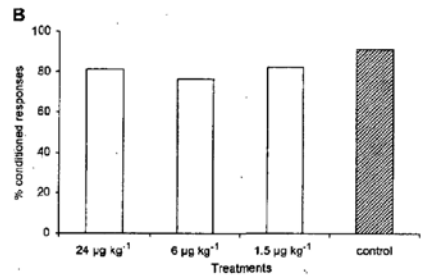
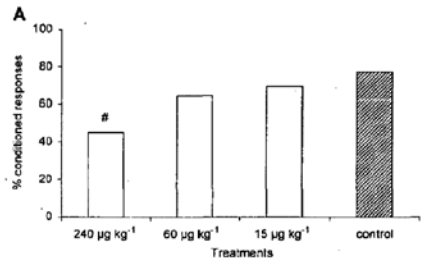




**Figure 2.** Experiment 1—effects of imidacloprid on learning performances in winter honeybees. (A) Bees exposed to a first set of three concentrations of imidacloprid (white bars; number of bees per group comprised between 27 and 38), and (B) bees exposed to a second set of three concentrations (number of bees per group was between 32 and 35). Control untreated groups (striped bar; number of bees per group was between 32 and 36) were included in each set. The number of conditioned responses at the test trial were compared between each concentration of the chemical and the control using a chi-squared test with 1 *df* (# $P < 0.0166$). When conditions of application of the chi-squared test were not fulfilled, the Fisher's exact method was applied.



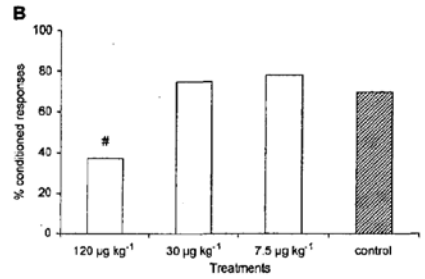
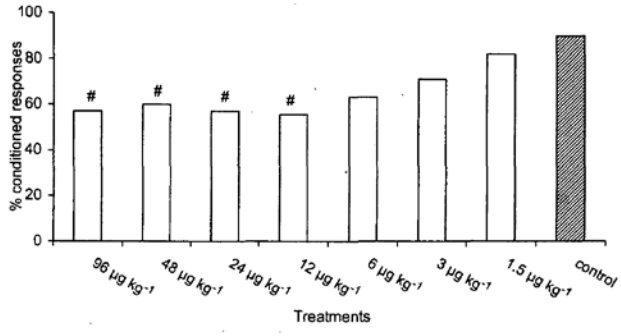




**Figure 3.** Effects of 5-OH-imidacloprid on learning performances in winter honeybees. (A) Bees exposed to a first set of three concentrations of 5-OH-imidacloprid (white bars; number of bees per group were between 20 and 36), and (B) bees exposed to a second set of three concentrations (number of bees per group comprised between 23 and 36). Control untreated groups (striped bar; number of bees per group were between 33 and 35) were included in each set. The number of conditioned responses at the test trial were compared between each concentration of the chemical and the control using a chi-squared test with 1 *df* (# $P < 0.0166$). When conditions of application of the chi-squared test were not fulfilled, the Fisher's exact method was applied.

**Figure 4.** Experiment 2—effects of imidacloprid on learning performances in summer honeybees. The number of bees per group was between 27 and 30. The number of conditioned responses at the test trial were compared between each concentration of the chemical (white bars) and the control (striped bar) using chi-squared test with 1 *df* (# $P < 0.0071$). When conditions of application of the chi-squared test were not fulfilled, the Fisher's exact method was applied.




**Description of Use in Document (QUAL, QUAN, INV):**

Qualitative

**Rationale for Use:** The lack of difference in consumption between the treatment groups and the control groups suggests the lack of antifeedant properties for honey bees at concentrations ranging from 1.5 - 96µg/kg for imidacloprid and 7.5 – 240 µg/kg for 5-OH-imidacloprid. The study also presents information on the sub-chronic dietary toxicity of imidacloprid and 5-OH-imidacloprid related to both summer bees and winter bees. The study reveals that concentrations of imidacloprid at 48µg/kg elicits significantly higher mortality to winter bees, whereas concentrations at 96 µg/kg to summer bees results in significantly higher mortality. This information suggests possible differences in sensitivity between summer and winter bees. 5-OH-imidacloprid appears to be less potent in terms of mortality than parent imidacloprid where concentrations at 240 µg/kg elicited significantly greater mortality, though there is uncertainty in this endpoint as noted below. In contrast, based on sublethal impacts to reflex response, both imidacloprid and 5-OH-imidacloprid produce an adverse effect at nearly identical concentrations. Both chemicals also impact the ability of winter bees to perform learned tasks at similar concentrations, though with parent imidacloprid being slightly more potent. The impact on learning performance from parent imidacloprid suggests that summer bees are more sensitive to sublethal impacts on learning, and winter bees are equally sensitive to mortality and sublethal learning endpoints.

**Limitations of Study:** The study did not employ negative controls, but only used positive controls containing acetone with which to compare the performance of the treatment groups. The study authors reported that they stored the stock solutions in the freezer, but removed them at ambient temperature and in daylight for defrosting even though imidacloprid will photodegrade. It is therefore possible that some degradation occurred prior to exposure to the test substance and may not be captured by the chemical analysis of the stock solution. The study did not present the raw data and so a review of the statistics could not be performed. The chronic mortality endpoint noted in the study for 5-OH-imidacloprid was shown to be 240 µg/kg. However, there was also substantial mortality of 17.2%. It is worth noting that the 850.3020 EPA guideline on honey bee contact toxicity testing states that mortality above 20% is considered to compromise the results of the study. The impacts on learning and reflex response present information on potential mechanisms for other apical endpoints; however, the study does not experimentally provide this linkage. Therefore, it is unclear as to how these sublethal impacts may affect the colony as a whole related to measurement endpoints of concern. In addition, the measurement endpoint of mortality to the individual presents useful information related to protection goals but retains uncertainty in how this individual mortality may impact whole colony hive dynamics. Finally, this study presented information on the learning performance of summer honeybees exposed to parent imidacloprid, which appeared to be a much more sensitive endpoint compared to mortality. Though it appears that 5-OH-imidacloprid is slightly less toxic relative to the parent compound concerning learning performance in winter bees, the study did not test what appears to be the more sensitive summer bees to 5-OH-imidacloprid exposure for this measurement endpoint.

**Primary Reviewer:**

Joseph DeCant, Ecologist, ERB5